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# THE C-PHYCOCYANIN/BETA PROTEIN INHIBITS CANCER CELL PROLIFERATION

by

HAIZHEN WANG

Under the Direction of Dr. Zhi-Ren Liu

## ABSTRACT

C-Phycocyanin (C-PC) from blue-green algae has been reported to have various pharmacological characteristics, including anti-inflammatory and anti-cancer activities. In this study, the  $\beta$ -subunit of C-PC (ref to as C-PC/ $\beta$ ) was expressed and purified from bacteria *E. coli* BL-21. The recombinant C-PC/ $\beta$  has been demonstrated to have anticancer properties. Under the treatment of 5  $\mu$ M of the recombinant C-PC/ $\beta$ , four different cancer cell lines accrued a high proliferation inhibition and apoptotic induction. The C-PC/ $\beta$  interacts with membrane-associated  $\beta$ -tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been found. Under the treatment of the C-PC/ $\beta$ , depolymerization of microtubulin and actin-filament was observed. The cells underwent apoptosis with increase of Caspase-3 and Caspase-8 activities. Cell cycle was arrested at G0/G1 phase under the treatment of C-PC/ $\beta$ . In addition, the nuclear level of GAPDH decreased significantly. Inhibition of cancer cell proliferation and induction of apoptosis may potentate C-PC/ $\beta$  as a promising cancer prevention or therapy agent.

Index Words:  $\beta$  subunit of C-Phycocyanin; Apoptosis; Cell cycle; Proliferation;

Caspase;  $\beta$ -tubulin; GAPDH

THE C-PHYCOCYANIN/BETA PROTEIN INHIBITS CANCER CELL PROLIFERATION

by

HAIZHEN WANG

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Masters of Science

in the College of Arts and Sciences

Georgia State University

2008

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2008

THE C-PHYCOCYANIN/BETA PROTEIN INHIBITS CANCER CELL PROLIFERATION

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## LIST OF ABBREVIATIONS

|                |   |
|----------------|---|
| aa             | Amino acid                                  |
| Brdu           | Bromo-deoxyuridine                          |
| BSA            | Bovine serum albumin                        |
| C-PC           | C-phycocyanin                               |
| C-PC/ $\alpha$ | Alpha subunit of C-phycocyanin              |
| C-PC/ $\beta$  | Beta subunit of C-phycocyanin               |
| CHCA           | Cyno-4-Hydroxycinnamic acid                 |
| DMEM           | Dulbecco's modified eagles media            |
| DNA            | Deoxyribonucleic acid                       |
| dNTP           | Deoxynucleotide triphosphate                |
| DMSO           | Dimethylsulfoxide                           |
| DTT            | Dithiothreitol                              |
| <i>E. coli</i> | <i>Escherichia coli</i>                     |
| EDTA           | Ethylenediaminetetraacetic acid             |
| FCS            | Fetal calf serum                            |
| GAPDH          | Glyceraldehyde 3-phosphate dehydrogenase    |
| GST            | Glutathione-S-transferase                   |
| HEK293         | Human embryonic kidney 293                  |
| HPLC           | High performance liquid chromatography      |
| IP             | Immunoprecipitation                         |
| IPTG           | Isopropylthiogalactoside                    |
| LB             | Lauria-Bertani                              |
| MALDI          | Matrix assisted laser desorption ionization |
| MS             | Mass spectrometry                           |
| MW             | Molecular weight                            |
| PAGE           | Polyacrylamid gel elektrophoresis           |
| PBS            | Phosphate buffer saline                     |
| PCR            | Polymerase chain reaction                   |

|       |  |
|-------|--|
| pH    | Potentia hydrogenii                          |
| PI    | Propidium iodide                             |
| PMSF  | Phenylmethanesulphonylfluoride               |
| RNA   | Ribonucleic acid                             |
| ROS   | Reactive oxygen radicals                     |
| rpm   | rounds per minute                            |
| RPMI  | Roswell Park Memorial Institute              |
| SCCHN | Squamous cell carcinoma of the head and neck |
| SDS   | Sodium dodecyl sulfate                       |
| TEMED | N,N,N',N'-tetramethylethylenediamine         |
| TNF   | Tumor necrosis factor                        |

## INTRODUCTION

### **C-Phycocyanin (C-PC) and its biological functions**

Blue-green algae or cyanobacteria belong to simple prokaryotes. They can be divided into edible and inedible species. Edible blue-green algae are nutrient-dense, and they are rich in proteins, vitamins, minerals, and essential fatty acids [Annapurna, 1991; Clement, 1975]. Studies showed the effects of blue-green algae on immunity [Amar, 2004; Teneva, 2005], anti-inflammatory response [Bruno, 2005], leukocyte trafficking [Mao, 2005], and processes anti-viral effects [Schaeffer, 2000], as well as anti-cancer functions [Khan, 2005]. Previous studies were based on the whole algae of various species treat to both human and animal studies, including *in vitro* studies on algae extracts and purified compounds.

Phycobiliproteins are water-soluble fluorescent proteins derived from blue-green algae and eukaryotic algae. In these organisms, they are used as accessory pigments for photosynthetic light collection. The energy is absorbed by phycobiliprotein in the visible spectrum that are poorly utilized by chlorophyll and, the energy is conveyed to chlorophyll at the photosynthetic reaction center. Phycobiliproteins are classified into two large groups based on their color, the phycoerythrins (red) and the phycocyanins (blue). The phycocyanins include C-Phycocyanin, R-Phycocyanin, and allophycocyanin. C-Phycocyanin (C-PC) is the major phycobiliprotein in many blue-green algae. The pigment has a single absorbance wavelength between 615 and 620 nm, and has a fluorescence emission at 640 nm. The molecular weight of this pigment is between 70,000 and 110,000 Da. Alpha and beta are the two subunits of the pigment. There are equal numbers of alpha and beta subunits. However, the numbers of alpha-beta pair vary among the different species. The intense blue color in blue-green algae is due to the phycocyanin.

Phycocyanin serves as a protein storage unit and as an antioxidant [Benedetti, 2004]. C-PC is widely used as natural dye for various purposes, due to its deep and intense color. C-PCs that come from different species, such as *Aphanizomenon* sp. [Benedetti, 2006], *Spirulina* sp. [Madhyastha, 2006], *Phormidium* sp. [Satyanarayana, 2005], *Lyngbya* sp. [Patel, 2005], *Synechocystis* sp. [Rogner, 1990], *Synechococcus* sp. [Bryant, 1990], have been isolated and studied.

### **Anti-cancer property of C-PC**

The pharmacological effects of C-PC include hepatoprotection [Vadiraja, 1998], antioxidation, anti-inflammatory [Romy, 1998], and radical scavenging [Bhat, 2001]. C-PC has been shown to prevent the growth of malignancy cancer *in vivo* [Dainippon Ink & Chemicals, 1983]. *In vitro* experiment showed that C-PC have the capability of inhibition the growth of cancer cells [Pardhasaradhi, 2003; Subhashini, 2004].

C-PC lowers the amount of cyclooxygenase-2 which is usually upregulated in cancer cells [Reddy, 2003]. It was shown that C-PC induces apoptosis by changing the Bcl-2/Bax ratio (Bcl-2, anti-apoptosis protein; Bax, pro-apoptosis proteins; the ratio of Bcl-2/Bax represents the degree of apoptosis) and the releases of cytochrome *c* into the cytosol. Phycocyanin induced apoptosis in AK-5 cancer cells involves down-regulation of Bcl-2 [Pardhasaradhi, 2003]. Furthermore, when the leukemia cell line was treated with C-PC, the cleavage of poly (ADP) ribose polymerase was enhanced [Subhashini, 2004]. Phycocyanin shows inhibitory effects on cancer necrosis factor-alpha activity [Romy, 2001; Ramirez, 2002]. C-PC induced Hela cell apoptosis was caspase-dependent. Caspases-2, -3, -4, -6, -8, -9, and -10 are involved under C-PC treatment [Li, 2006]. Percentage of cells arrested in sub-G0/G1 phase was increased under the treatment with C-PC [Reddy, 2003; Subhashini, 2004; Li, 2006]. The activity of C-PC in the

induction of cancer cell apoptosis and its relatively low-toxicity of non-cancer cells make it a very good candidate as a cancer prevention agent. However, the molecular mechanism underlying the anti-cancer activities of C-PC is not well understood.

### **Apoptosis and cell cycle**

Apoptosis, also called programmed cell death, is the mechanism complementary to proliferation. When cells undergo apoptosis, several classical phenomena can occur, including membrane blebbing, condensation of cytoplasm and the activation of endonuclease/ specific proteases. Later on, the genome will be fractured, and smaller apoptotic bodies will be formed [Bedner, 1999]. Macrophages will phagocytose the apoptotic cell. Through the process of apoptosis, unwanted or dysfunctional cells can be eliminated. Two major apoptosis pathways have been demonstrated. One is the mitochondrial/cytochrome C (intrinsic) pathway, which activates Caspase-9 and Caspase-3. The second pathway is extrinsic pathway, in which extrinsic signals activate Caspase-8 and Caspase-3. Caspase-3 is activated in most of the process of apoptosis [Creagh, 2003]. Caspase-3 is the most important protein in the process of apoptosis. Caspase-3 will finally induce the apoptotic morphologies, including DNA fragmentation and cell shrinkage [Johnson, 2000].

The function of the cell cycle is to duplicate DNA and proteins and then separate the copies into two daughter cells. The cell grows in interphase, which including S phase (DNA replication), G1 phase (the gap between M phase and S phase), and G2 phase (the gap between S phase and M phase). In M phase, the nucleus and the cytoplasm divide into two daughter cells. Cell-cycle process can be arrested due to the intracellular or extracellular signals [von Wangenheim, 1998].

### **Tubulin and Glyseraldehyde-3-phosphate dehydrogenase (GAPDH)**

Tubulin is the major protein component of microtubules. Microtubule system play important roles in vesicle transport, chromosome segregation and maintain cell structure. Tubulin is one of the intracellular molecular targets of many anticancer drugs [Hadfield, 2003; Fojo, 2005]. Drugs like Taxel induce mitotic arrest and apoptosis in cancer cells through disrupting the dynamics of tubulin [Loganzo, 2003].

GAPDH functions as a glycolytic enzyme within the cytoplasm [Tisdale, 2004]. GAPDH also plays a role in other cellular processes including in membrane fusion [Han, 1998], microtubule bundling [Sirover, 1999], phosphotransferase activity [Cooper, 1998], nuclear RNA export [Kimura, 2000], DNA replication and DNA repair [Tatton, 2000]. It is also thought to be involved in cell apoptosis [Tatton, 2000]. Abnormal expression [Tachibana, 2003], nuclear accumulation [Senatorov, 2003], loss of enzymatic activity [Chuang, 2005], and changes in physical properties of GAPDH were found to associated with several diseases [Chuang, 2005]. GAPDH was suggested to be a potential molecular target for drug development.

### **Purification methods and physical property of C-PC**

To dates, all the previous studies used C-PC that was isolated from algae. The purification involved a multi-step treatment of the crude extract, including fractional precipitation with ammonium sulfate and separation over an ion-exchange chromatography column [Patel, 2005] or a hydroxyapatite column [Benedetti, 2006], or a gel filtration chromatography [Minkova, 2003].

Two components exist in many protein toxins: one component is responsible for the enzymatic activity; the other component is utilized to translocate the enzymatic component into the cells. The enzymatic activity component is released from the other component and exhibit

toxicity. C-PC is a kind of toxin to cancer cells, though it is a non-toxic protein to normal cells. The two subunits ( $\alpha$  and  $\beta$ ) form a heterodimer, which is very stable. Purified PC monomers can form trimers, and two trimers can assemble into a hexamer [Saxena, 1988]. The pairs of  $\alpha$  and  $\beta$  subunits of C-phycocyanins have the same 3D structure; however, their sequence is different [Glauser, 1992].

### **Specific Aims for this study**

- (i) Determine whether the recombinant beta-subunit of C-phycocyanin has the anti-cancer properties (different cancer cell lines were used).
- (ii) Identify how the recombinant beta-subunit of C-phycocyanin inhibits the cancer cell growth.

### **Choice of cell lines**

In this study, six cell lines were used. Four of the cell lines are cancer cells, and two of the cell lines are non-cancer cells. Respectively, 686LN was derived from a squamous cell carcinoma of the head and neck patient's metastatic tumor, 686LN-M4C1 was derived from 686LN cells passaged four times in nude mice; K562 is chronic myelogenous leukemia cells, and HT 29 is colorectal adenocarcinoma; and two normal cell lines were used: C5/MJ is T lymphocyte, RPMI 1788 is B lymphocyte. K562 was used to determine the appropriate dosage of recombinant C-PC/ $\beta$  protein for the treatment. 686LN-M4C1 cell was used as the main study system in order to figure out the anti-cancer mechanism of recombinant C-PC/ $\beta$  protein.

### **Overview of this study**

In the present study, the recombinant C-PC/ $\beta$  protein was successfully obtained. The optimum treatment dosage of the recombinant protein was determined based on the experimental result using K562 cells (human chronic myeloid leukemia cells). The cell lines used in this study



are: 686 LN (poorly metastatic parental head and neck cancer), 686LN-M4C1 (metastatic head and neck cancer), HT 29 (Colorectal adenocarcinoma) and normal cell lines: C5/MJ (T lymphocyte), RPMI 1788 (B lymphocyte). It is evident that the recombinant C-PC/ $\beta$  is an inhibitor for cancer cell proliferation and an inducer for cancer cell apoptosis. The C-PC/ $\beta$  inhibits cancer cell proliferation and promotes cancer cell apoptosis. The protein was the most effective in 686 LN-M4C1 cells. Therefore this cell line was chosen to further study the molecular mechanism of the effects. It showed that the protein depolymerized microtubule/microfilament and affected GAPDH redistribution. The protein also arrested the cell cycle at G0/G1 phase, and activated Caspases -3 and -8. This study demonstrated a new insight into the molecular mechanism of anti-tumor activity of C-PC. More importantly, this study demonstrated that the  $\beta$  subunit of C-PC has much higher anti-tumor activity, and the molecular mechanism of anti-cancer appears different from that of the whole C-PC. On the other hand, both tubulin and GAPDH are the targets by the C-PC/ $\beta$ . The recombinant C-PC/ $\beta$  protein shows potential in the treatment of metastatic head and neck cancer.

## MATERIALS AND METHODS

### Whole cell polymerase chain reaction (PCR) and plasmid construction

Blue-green algae or Cyanobacteria are Eubacteria. They have a rigid cell wall but no mitochondria or other large organelles, and they have a single chromosome and the chromosome exists as a linear or circular strand. Whole cell PCR is a method that can be used to obtain the target DNA sequence without the need for purification of genomic DNA of the strain of interest. This method is quite suitable to obtain the DNA sequence of Eubacteria, in that the unique genomic DNA structure of the Eubacteria has no intron DNA sequences and the genomic DNA is loosely packed. *Nostoc* PCC 7120 is one common strain of blue-green algae. Therefore, we applied whole cell PCR to get the DNA fragments of  $\beta$  subunit of C-PC.

The published gene sequence of  $\beta$  subunit of C-PC was obtained from [www.Pubmed.com](http://www.Pubmed.com) (Accession No. X05239). Living alga, *Anabaena* PCC 7120 was obtained from the culture collection of algae at the University of Texas at Austin. The *Anabaena* pellet was centrifuged and collected when the alga reached the exponential growth phase in BG11 medium. The alga was washed with distilled water, pelleted again in preparation for PCR. Primers used to amplify the C-PC/ $\beta$  were: forward 5'-GGGGATCCATGACATTAGACGTATTTA-3' and reverse 5'-GGGAATTCTTTAA CCAACAGCAGCAGCAG-3'. 50  $\mu$ l of PCR mixture consists of: algae pellet 1  $\mu$ l, forward primer and reverse primer 0.3  $\mu$ l each with the concentration (about 0.4  $\mu$ g/ $\mu$ l), dNTP mixture 0.5  $\mu$ l (10mM), Pfu DNA polymerase 1  $\mu$ l, Pfu buffer 5  $\mu$ l, and distilled water 41.9  $\mu$ l. PCR amplification was carried out for 35 cycles under denaturing –annealing –extension conditions of 94 °C for 30 seconds, 55 °C for 1 minute, 65 °C for 4 minutes respectively.

pGEX-2T is an expression vector for fusion proteins. It encodes a glutathione S-transferase gene at the N-terminal of the fused protein. A thrombin recognition site exists between the GST and the fused protein. The pGEX vectors contain a tac promoter. The promoter can be induced to express the recombinant protein with 1-5 mM IPTG. The expressed fusion proteins will contain the 26 kDa glutathione S-transferase (GST). An ATG and ribosome-binding site are included in the GST gene, which is under control of the tac promoter. A translation terminator exists in each reading frame.

The PCR product of amplified DNA fragment coding the subunit of C-PC was obtained. The product was cloned into SmaI-digested pGEM-4Z (cloning vector). Then the insert was sub-cloned into the BamHI and EcoRI sites of pGEX-2T, yielding plasmids pGEX-2T- $\beta$ . The  $\beta$  subunit of C-PC expressed from pGEX-2T- $\beta$  consists of the  $\beta$  subunit fused at the C terminus to a GST tag, in which includes a thrombin cleavage site. The constructed plasmid was verified via DNA auto-sequencing.

#### **Analysis of $\beta$ subunit of C-PC based on the amino acid sequence**

Transmembrane prediction and Protparam (Swiss-Prot/TrEMBL) soft analysis on the website was performed to analyze the theoretical molecular weight, pI, hydrophaticity, transmembrane helices predicted, and stability level of the proteins (GST, GST:C-PC/ $\beta$ , and  $\beta$ ). The amino acid sequences of proteins were used. Based on the analysis results, we applied the corresponding pH value when purifying or dialyzing the proteins we expressed.

#### **Transformation of the constructed plasmid into the expression model *E. coli* BL-21**

5 ml of LB medium with Ampicillin (100 mg/ml) was pre-warmed to 37°C. Bacterial *E. coli* BL-21 was thawed on ice. 100  $\mu$ l of bacteria was aliquoted into 1.5 ml eppendorf tube. 100 ng of plasmid DNA was added to the tube, and the mixture was swirled softly and incubated on

ice for 30 minutes. Then the tube was placed in the water bath at 42 °C for 90 seconds (heat shock). After that, the tube was placed on ice again for 2 minutes. Then 200 µl of pre-warmed LB with Ampicillin was added to the tube and the tube was shaken (225 rpm) for 1 hour at 37 °C. Later on, 100 µl of the transformed *E. coli* was pipetted to pre-warmed LB agar plates (Ampicillin was added), and spread with a bacterial spreader. The plates were incubated at 37 °C until the solution was absorbed, and were turned upside down and incubated overnight at 37 °C. The following morning, one single colony was picked up for culture and induction.

### **Expression and purification of the Recombinant $\beta$ subunit of C-PC**

GST-fusion proteins can bind to glutathione-Agarose. Therefore, a high degree of purification of fusion protein can be achieved in just one affinity purification step. Tag-free recombinant proteins can be obtained through removing the fusion tags by treatment with enzymes. pGEX-2T allows for removal of the GST carrier protein from the fusion protein by thrombin cleavage.

The constructed plasmids, pGEX-2T-  $\beta$  was transformed into *E. coli* BL-21 codonplus®, and cultured in LB medium containing Ampicillin to  $A_{600nm}=0.6$ . The expression of the recombinant proteins was induced by 0.1 mM IPTG for 4.5 hours. 1 ml of culture solution was collected and centrifuged, and the cell pellet was washed with PBS. The pellet was resuspended in 100 µl 1×SDS-PAGE loading buffer, and heated to 95~100 °C for 10 minutes. A 10% SDS-PAGE gel was used to examine the expression of the protein. Additionally, cell pellets were thawed and resuspended in lysis buffer (1% N-lauroyl sarcosine, 1 mM EDTA, 500 µM PMSF, 10 mM DTT in PBS, pH 7.3, pre-cooled to 4 °C). The cell solution was sonicated for 3 minutes and centrifuged at 15,000 rpm for 20 minutes. The supernatant was collected and filtered using a 0.45 µm syringe filter. The filtered supernatant was added to the glutathione-agarose and

incubated at 4 °C for 40 minutes with shaking. Then, a column was used to collect the beads and the solution was filtered once more. The resin was washed with PBS-T (1% Triton X-100 and 1mM DTT) 4 times. GST-  $\beta$  was eluted and collected (10mM reduced glutathione in 50mM Tris-HCl, pH 9.45) during this step. 40 units of thrombin were diluted into 3 ml PBS containing 1 mM DTT. The  $\beta$  subunit of C-PC were cleaved overnight using the protease solution. The beads were washed and elution buffer was used to collect the GST protein.

HiLoad 16/60 Superdex 200 is used for gel filtration. Gel filtration is a liquid chromatography technique that separates molecules according to their size. The selectivity curve is usually fairly straight over the range  $K_{av}=0.1$  to  $K_{av}=0.7$ , which shows in Graph 1. The molecular weight range, which lies between these values, is defined as the useful fractionation range of the medium.

According to Graph 1, the proteins with a molecular weight between 30,000 Da–600,000 Da MW are high on the selectivity range for Superdex 200 prep grade. The molecular weight of GST- $\beta$  and  $\beta$  are within this range, therefore, we used Sephadex 200 gel-filtration column to further purify the proteins (GST tagged  $\beta$  subunit of C-PC and GST).

Centriprep Centrifugal Filter Devices were applied to concentrate the GST tagged  $\beta$  subunit of C-PC and GST recombinant proteins ( $3 \times 1000g$ , 4 °C).

### **Refolding and dialysis of the protein $\beta$ and GST- $\beta$**

One liter of PBS (pH 7.3, pre-cooled to 4 °C) was prepared, which is over 50 times of the volume of the sample. DTT was added to a final concentration 0.1 mM. The protein sample was dialyzed for at least 3 hours at 4 °C. The buffer was changed (same components) and the sample was dialyzed for an additional 3 hours. Finally, the protein sample was dialyzed through two additional round with buffer without DTT. A dialysis buffer containing 1mM reduced

glutathione and 0.2mM oxidized glutathione in  $1 \times$  PBS (over 25 times volume of sample) was prepared and chilled to 4 °C. The refolded protein was dialyzed overnight at 4 °C (if disulfide oxidation or isomerization required, continued with this step). Then the protein sample was dialyzed for another 3 hours in PBS only and the protein activity was assayed.

### **Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE is the most commonly used protein electrophoresis method. First, SDS and a reducing agent such as DTT are added on a weight ratio basis: 1.4 mg SDS per 1 g protein, and temperature is elevated to over 100 °C to denature the proteins. The denatured proteins are wrapped with SDS molecules. The proteins are mainly separated according to sizes of proteins regardless of the charges. Theoretically, SDS-PAGE can separate proteins from around 10,000 Da to 300,000 Da molecular weights by varying pore sizes of the gels. The pore size of the gel can be varied by adjusting the ratio of the acrylamide, cross-linker, and bisacrylamide. SDS-PAGE can be used for assessing purity and as a tool to primarily determine the molecular weights by comparing the unknown sample with the known protein. To separate the protein bands, silver- or Coomassie Blue-staining methods can be used to visualize the proteins. In this experiment, 10% or 14% gels were used to show the expression level, the molecular weight, and the purity of the proteins.

### **Protein Mass Spectrometry.**

Because the protein samples in SDS-PAGE are denatured, its application in detecting conformational change is limited. Here, we further use the protein mass spectrometry to show the molecular weight and the purity level of the proteins.

The samples were dialyzed into 10 mM Tris-HCl buffer with the same pH value of 7.3, concentrated by vacuum centrifugation, and subsequently applied to ZipTip C18 tips for

desalting and concentrating. After ZipTip purification, the proteins were eluted from the ZipTip with a Cyano-4-Hydroxycinnamic acid (CHCA) solution in 50% acetonitrile (CAN)/ 0.1% formic acid and spotted directly onto a wax-coated matrix-assisted laser desorption/ionization target plate. Protein mass spectrometry analysis was applied to the samples.

### **Cell culture and protein treatment**

Cell proliferation was determined using the MTT assay. Human chronic myeloid leukemia K562 cells ( $2 \times 10^4$ / well, 96-well plate) with fresh RPMI 1640 medium (10% FCS) were first treated with 0.44  $\mu$ M and 4.4  $\mu$ M recombinant  $\beta$  subunit of C-PC (PBS buffered, pH 7.3), and PBS buffer treatment was used as a control. 686LN-M4C1, 686LN, K562, HT 29, C5/MJ and RPMI 1788 cells were seeded on plate ( $2 \times 10^4$ / well, 96-well plate) with appropriate fresh media. Adherent cells were seeded for 24 hours, the medium was then changed on the second day prior to treatment. The suspension cells were inoculated on the same day. 5  $\mu$ M of the recombinant  $\beta$  subunit of C-PC protein was used to treat the different cell lines. All the proteins added were PBS buffered (pH 7.3). 5  $\mu$ M of GST and PBS were used as two controls. Morphological changes were examined using light microscopy (100 $\times$ ) daily, for three days. Cultures were maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37 $^{\circ}$ C for 48 hours.

### **MTT assay method**

In this experiment we use CellTiter 96® non-Radioactive Cell Proliferation Assay to assay the activity of the C-PC/ $\beta$  on the different cell lines.

In our experiment, after treatment, an MTT assay: CellTiter 96® non-Radioactive Cell Proliferation Assay was performed. 15  $\mu$ l of the Dye Solution was added to each well. The plate was incubated at 37 $^{\circ}$ C for up to 4 hours in a humidified, 5% CO<sub>2</sub> atmosphere. After incubation, 100  $\mu$ l of the Solubilization/Stop Solution was added to each well. One hour after addition of the

Solubilization/Stop Solution, the contents of the wells were mixed to obtain a uniformly colored solution. The absorbance values at 570 nm were determined on a multi-well plate reader. The assay was performed on samples and controls in triplicate.

#### **Apoptosis detection of protein treated cancer cells (Cell Death Detection ELISA<sup>PLUS</sup>)**

686LN-M4C1, 686LN, K562 and HT 29 cells were seeded ( $2 \times 10^5$ / well, 12-well plate) with corresponding fresh medium. The same concentration of the recombinant  $\beta$  subunit of C-PC protein (5  $\mu$ M) was applied to all cell lines. 5  $\mu$ M of GST and PBS treatment were used as two controls. On the third day, the same amount of treated and untreated cells ( $5 \times 10^4$  cells) per the Cell Death Detection ELISA<sup>PLUS</sup> were counted and collected. The cells were washed with pre-cooled PBS, centrifuged and resuspended in 200 $\mu$ L of lysis buffer. The cells were lysed for 30 minutes at room temperature, centrifuged at 200 g for 10 minutes. 20  $\mu$ L of supernatant solution from each control was transferred to Streptavidin-coated microplates and shaken for 2 hours at room temperature. The solution was removed and rinsed with incubation buffer. 100  $\mu$ L substrate solution was added and incubated until the color development was sufficient for a photometric analysis at 405 nm using the substrate solution as a control blank. The specific enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated from these values using the following formula: enrichment factor= absorbance of the sample (treated) / absorbance of the corresponding negative control (non-treated).

#### **Caspases activation detection**

Caspases exist in inactive form and active form. Only when the regulatory domain is cleaved, Caspases can be activated. The status of caspases can be detected via immunoblots method with specific antibodies. 686LN-M4C1 cells were inoculated into 6-well dishes with corresponding fresh medium. The same concentration of the recombinant  $\beta$  subunit of C-PC



protein (5  $\mu$ M) was added to the cells, PBS treatment was used as a control. Cells were collected 24 hours after addition of the protein. 1 $\times$ RIPA buffer supplemented with protease inhibitors was used to prepare whole cell lysates. 50  $\mu$ g of the protein was used for the immunoblotting. A 14% SDS-PAGE gel was utilized to separate the proteins. The immunoblots were probed with appropriate antibodies: mouse monoclonal antibodies of anti-Caspase-3, anti-Caspase-8 and rabbit monoclonal antibody of anti-cleaved-Caspase-3.

### **Fluorescence Activated Cell Sorting (FACS) analysis of cell cycle**

686LN-M4C1 cells were inoculated into 6-well dishes with corresponding fresh medium. The same concentration of the recombinant  $\beta$  subunit of C-PC protein (5  $\mu$ M) was added to the cells, PBS treatment was used as a control. After the above treatment (48 hours),  $1\sim 2\times 10^6$  cells were harvested and washed in PBS, then fixed with 1% (w/v) paraformaldehyde (methanol free) and placed on ice for 15 minutes. After the cells were washed with pre-cooled PBS, they were resuspend in 5 ml of ice-cold 70% (v/v) ethanol. The cells stood for 30 min on ice or cell solution was stored in the freezer (-20 $^{\circ}$ C) until the next step. Cells were stained with 0.5 ml PI/RNase staining buffer (51-6585AZ, BD Bioscience) for 15 minutes at 37 $^{\circ}$ C and analyzed by FACS (Becton Dickinson) (FL2-A).

### **Immunofluorescent confocal microscopy**

686LN-M4C1, 686LN, K562, and HT 29 cells were seeded into culture wells ( $4\times 10^4$ /well, culturewell<sup>TM</sup> chambered cover-glass for cell culture) with corresponding fresh media. 5  $\mu$ M of the recombinant  $\beta$  subunit of C-PC protein was added to the cells. On the third day, a general fixation protocol was used to prepare the samples as follows: the cells were rinsed with pre-warmed buffer and fixed with 3.7% formaldehyde; the cells were rinsed and permeabilized in 0.1% Triton X-100; the cells were blocked with Image-iT<sup>TM</sup> FX signal enhancer; the samples

were incubated with the GST antibody in 1:50 dilution; the cells were washed and incubated with second detection conjugates (Alexa Fluor 488 goat anti-mouse IgG (H+L) in 1: 200 dilution; the samples were mounted with Prolong Gold anti-fade reagent, and allowed to cure until ready to examine via laser scanning confocal microscope (Zeiss LSM 510). The above method was applied to the adherent cells; however, for suspension cells, centrifugation at 1,000 g at 4 degree Celsius for 10 minutes was used to replace the medium, buffer and reagents.

### **Membrane protein extraction**

Mem-PER® Eukaryotic Membrane Protein Extraction Reagent Kit was used to obtain the membrane protein extracts.  $5 \times 10^6$  cells were obtained by centrifuging harvested 686LN-M4C1 cell suspensions at  $850 \times g$  for 2 minutes. Cells were washed by PBS and were pelleted in 1.7 ml microcentrifuge tubes. The supernatant was carefully removed and discarded. 150  $\mu$ l of Reagent A was added to the cell pellet. A homogeneous cell suspension was obtained by pipetting up and down. The suspension was incubated 10 minutes at room temperature with occasional vortexing. The lysed cells were placed on ice. One part Reagent B was diluted with two parts Reagent C. Reagents B and C were kept at 4°C or on ice at all times. 450  $\mu$ l of diluted Reagent C was added to each tube of lysed cells and vortexed. The tubes were incubated on ice for 30 minutes, vortexing every 5 minutes. The tubes were then centrifuged at  $10,000 \times g$  for 3 minutes at 4°C. The supernatant was transferred to new tubes and incubated for 10 minutes at 37°C to separate the membrane protein fraction. The tubes were centrifuged at room temperature for 2 minutes at  $10,000 \times g$  to isolate the hydrophobic fraction from the hydrophilic fraction. The hydrophilic phase (top layer) was carefully removed from the hydrophobic protein phase (bottom layer) and saved in a new tube. The separated fractions were placed on ice. The majority of

membrane proteins should be in the lower hydrophobic fraction, which was used for membrane protein analysis.

The detergent in the membrane fraction was reduced through dialysis so that the sample was compatible with the pull-down experiment. The sample was dialyzed overnight at 4°C against a buffer that contains 0.5% detergent (NP-40) to maintain protein solubility as buffer exchange occurs. A volume that was 150-fold greater than the volume of the sample was used to dialyze and the dialysis buffer was changed twice.

### **Pull-down assay**

The GST tagged C-PC/β was pre-bound to the glutathione-agarose beads. The GST moiety and the column were used as controls. The beads were incubated with the membrane protein extracts prepared as above. The agarose beads were washed 5 times with PBS buffer containing 0.5% NP-40. The GST pull-down proteins were separated by SDS-PAGE. The gels were stained with the gelcode® blue stain reagent (Pierce).

### **Associated protein identification (TOF/TOF-MS)**

The identified protein bands (pulled-down by the C-PC/β) were sliced out and placed in siliconized eppendorf tubes. The gel piece was covered with 200 µl of Destaining Solution and incubated at 37 °C for 30 minutes. The solution was removed and discarded from the tube (repeated 2 times). The gel piece was dried in a speed vacuum for approximately 30 minutes. 20 µl (0.4 µg of trypsin) of the prepared Trypsin Solution was added to the gel sample. 50 µl of the Trypsin Reaction Buffer was added to the gel sample and the sample was incubated for 4 hours at 37 °C. After the incubation, the liquid was removed from the gel piece and the liquid was transferred to a new tube. This solution contained the extracted tryptic peptides, which were ready for TOF/TOF-MS analysis. The mass spectrometer first obtained MS spectra of each of the

samples followed by MS/MS analysis of the 25 most abundant peptide peaks from each spectrum. The combined MS and MS/MS data was used to search the NCBI database. Peptides with “ion scores” were fragmented by MS/MS and ion scores above 30 were considered significant.

### **Coimmunoprecipitation (Co-IP) to further prove the interaction of the protein**

The GST tagged C-PC/ $\beta$  was pre-bound to the glutathione-agarose beads. The GST moiety and the column were used as controls. The beads were incubated with the membrane protein extracts prepared as above. The agarose beads were washed 5 times with PBS buffer containing 0.5% NP-40. The GST pull-down proteins were separated by SDS-PAGE. The pull-down proteins were further analyzed by immunoblot using antibodies against  $\beta$ -tubulin and GAPDH.

30 $\mu$ L of Immunopure Protein A agarose (capture reagent) which was equilibrated to 50% in the appropriate wash buffer (equilibration: 3 washes with wash buffer for IP) was prepared. The first antibodies (polyclonal antibody against GAPDH or polyclonal antibody against  $\beta$ -tubulin) and capture agent were pre-coupled separately (The mixture were incubated for 2 hours at 4 °C, and washed by PBS buffer containing 0.5% NP-40 for three times). The pre-coupled agarose was centrifuged and then incubated with the membrane protein extracts for 4 hours at 4 °C. The beads and associated proteins were centrifuged and washed for 5 times. Loading buffer was added to the beads, and followed by vortexing, boiling, and running SDS-PAGE gels. The interactions of C-PC/ $\beta$  with  $\beta$ -tubulin and GAPDH were shown by co-immunoprecipitates, which followed by immunoblot using antibodies against  $\beta$ -tubulin, GST and GAPDH, respectively.

### **Nuclear and cytoplasmic protein extracts**

Cells were gently removed from dish by scraping with cell lifter. Cells were washed with ice-cold PBS with phosphatase inhibitors and the cell suspension was centrifuged for 5 minutes at 500 rpm in a centrifuge pre-cooled at 4°C. This step was repeated three times. The supernatant was discarded and the cell pellet was placed on ice. The cell pellet was gently resuspended in 500 µL 1X Hypotonic Buffer by pipetting up and down several times. The suspension solution was transferred to a pre-chilled microcentrifuge tube and was incubated for 15 minutes on ice. 25 µL of detergent was added and the sample was vortexed for 10 seconds at the highest setting. The suspension solution was centrifuged for 30 seconds at 14,000 x g in a microcentrifuge pre-cooled at 4°C. The supernatant (cytoplasmic fraction) was transferred into a pre-chilled microcentrifuge tube and stored at -80°C until ready to use. The nuclear pellet was resuspended in 50 µL Complete Lysis Buffer by pipetting up and down. The sample was vortexed for 10 seconds at the highest setting. The suspension solution was incubated for 30 minutes on ice on a rocking platform set at 150 rpm. Then the sample was vortexed for 30 seconds at the highest setting. The sample was centrifuged for 10 minutes at 14,000 x g in a microcentrifuge pre-cooled at 4°C. The supernatant (nuclear fraction) was transferred into a pre-chilled microcentrifuge tube and stored at -80°C.

### **Soluble and assembled tubulin / actin measurement**

Soluble (depolymerized) tubulin / actin and assembled (polymerized) tubulin /actin were measured as described previously [Ogawa, 2002]. Briefly, 686LN-M4C1 cells were treated with PBS or the recombinant  $\beta$  subunit protein for one day. After treatment, cells were washed with PBS containing 0.5 µg/ml Paclitaxel, and lysed with 100 µL of lysis buffer (20mM Tris-HCl, 0.5% NP-40, 1mM MgCl<sub>2</sub>, 2mM EGTA, and 0.5 µg/ml Paclitaxel). Then the samples were

vortexed and centrifuged at  $12,000\times g$  for 10 min at  $4^{\circ}\text{C}$ . Supernatants containing soluble tubulin / actin were separated from pellets containing polymerized tubulin /actin and placed in separate tubes. 100 $\mu\text{l}$  of water was used to wash the pellets. Lysis buffer was used to dissolve the pellets. A 14% SDS-PAGE gel was utilized to separate the proteins and immunoblots were probed with appropriate antibodies (antibody of  $\beta$ -tubulin, antibody of actin). The band of each sample was quantified through the use of an imaging program (Biochemi System), and the ratios of depolymerized to polymerized tubulin/actin were calculated for each treatment condition.

### **Expression level and subcellular distribution of GAPDH**

The western blot technique was used to detect the distribution of the GAPDH. 686LN-M4C1 cells were seeded into 6-well dishes with corresponding fresh medium. The same concentration of the  $\beta$  subunit of C-PC protein ( $5\text{ }\mu\text{M}$ ) was applied to the cells, and PBS treatment was used as the control. Cells were collected 24 hours after the addition of the protein. The cells were washed with pre-cooled PBS and a Nuclear Extraction kit was used to isolate the nuclear located proteins (The detailed steps were described previously). 115  $\mu\text{g}$  of the nuclear proteins was used for immunoblotting to detect the distribution of the GAPDH. Meanwhile, 50  $\mu\text{g}$  of the whole cell lysate proteins was used to detect the expression level of GAPDH under treatment. A 14% SDS-PAGE gel was utilized to separate the proteins and immunoblots were probed with appropriate antibodies: mouse monoclonal antibodies of anti-GAPDH.

Tubulin is one of the housekeeping genes. From the primary result, it showed that the expression level of tubulin did not changed significantly. Therefore, we used tubulin as our loading control. Histone 2A, exists inside of the nucleus. Here we used it as a control to show that there was no contamination of cytoplasmic extracts from nucleus.

50 µg of the cytoplasm proteins and nuclear proteins were used for the immunoblots of  $\beta$ -tubulin and Histone H2A. Monoclonal antibody against  $\beta$ -tubulin and polyclonal antibody against Histone H2A were used.

### **Statistical analysis**

The results were expressed as mean  $\pm$  S.D. of data obtained from three independent experiments. Statistical analysis of difference was carried out by analysis of variance (ANOVA). The level of significance was set at  $P < 0.05$ . (The analysis method was same used in the following experiment.)

## RESULTS

### Whole cell PCR product and plasmid were obtained

Whole cell PCR is quite suitable to clone the C-PC DNA sequence from *Nostoc PCC* 7120 (Figure 1). PCR products also were blunt ligated into the *Sma* I digested pGEM-4Z (Figure 2a), and sub-cloned into pGEX-2T (Figure 2b). We single-enzyme-digested the vectors, used the corresponding empty vector as a control, and compared the molecular size to test whether the vector has been cloned with the DNA sequence. We succeeded in obtaining the vector containing the DNA sequence. The constructed plasmid was sent for sequencing.

### Theoretical analysis results of corresponding proteins

Amino acid sequences of subunits of C-PC and GST are available from the PubMed, or we can decode the corresponding DNA sequences into the amino acid sequence (Table 2.1). Through Modern Transmembrane Prediction and Protparam (Swiss-Prot/TrEMBL) soft analysis, we found that the theoretical molecular weight of GST is around 28165.7 Da, GST- $\beta$  is around 46678.6 Da (Table 2.2). All the proteins were determined to be stable.  $\beta$  subunit of C-PC shows higher hydropathicity and no transmembrane helices were predicted. It shows that the theoretical pI of  $\beta$  is 5.00. At the same time, the grand average of hydropathicity (GRAVY) is -0.025 ( $\beta$ ). The estimated half-life of two subunits is around 30 hours applied to mammalian cells *in vitro*. According to the pI of the proteins and our study purpose, we decided to use PBS with the pH 7.2 ~ 7.4 to purify and dialyze all the proteins.

### High level expression and high purity of recombinant subunits of C-PC

The DNA fragments were cloned into the expression vector pGEX-2T and the subunits of C-PC were subsequently expressed in *E. coli* BL-21 as GST-fusion proteins. High expression level of C-PC/ $\beta$  was observed (Figure 3). After purification over a glutathione-agarose beads



column and a sephedex 200 gel-filtration column, high purity and concentration of the recombinant proteins were obtained (Figure 4). The concentration of the C-PC/ $\beta$  protein was estimated to be 0.9 mg/ml.

### **Effective refolding and dialysis of the recombinant proteins**

Since the  $\beta$  subunit contained three Cysteine amino acids, the protein can form disulfide bonds with itself through refolding and dialysis. Two bands on the SDS-PAGE gel were obtained when the refolded protein sample was treated by the loading buffer without reducing reagent (Figure 5, lane 1). While only one band on the SDS-PAGE gel was observed when the protein sample was treated with the loading buffer containing the reduce reagent (Figure 5, lane 2). The band which runs faster is reasoned to be the protein formed the disulfide bond, while the other one is linear protein (Figure 5, lane 1). In the following experiment, we used the proteins that were refolded and dialyzed and which showed highly toxic activity to cancer cells.

### **MS results were consistent with the theoretical molecular weights of the proteins**

Molecular weights of obtained recombinant proteins were determined by MALDI-TOF mass spectrometry (Figure 6). The mass spectrometry results indicated that the obtained GST:C-PC/ $\beta$  had MW 45.4 kDa and the GST had MW 27.1 kDa respectively. These molecular weights were consistent with the corresponding calculated MWs. The results also reflected that the proteins we got were in high level of purity.

### **The C-PC/ $\beta$ inhibits cell proliferation**

C-PC was reported to inhibit cell proliferation in a number of cancer cell lines [Pardhasaradhi, 2003; Subhashini, 2004]. We first determined the appropriate dosages of C-PC/ $\beta$  on human chronic myeloid leukemia cell line, K562. The proliferation rates of cells under the treatment of 0.44  $\mu$ M and 4.4  $\mu$ M of C-PC/ $\beta$  were around 98% and 60.8%, respectively (PBS

treatment was set as 100%) (Figure 7). Based on this results, we determined the optimum dose of the protein to the K562 cell was around 5  $\mu$ M. We applied this concentration of the protein to study the pharmacological effects in the following experiment. To test whether the recombinant C-PC/ $\beta$  also has the ability to inhibit the growth of different cancer cells, we carried out the MTT assay with a number of cancer cell lines and two normal cell lines: 686LN, 686LN-M4C1, K562, HT 29, C5/MJ and RPMI 1788. The same concentration of GST protein and PBS were used as controls. Growth of all cancer cells was largely inhibited by treatment with C-PC/ $\beta$ . The greatest inhibitory effect on the cell proliferation occurred to 686LN-M4C1 cells, which the growth proliferation of the cells was 39.6% (PBS treatment was used as a control). On the other hand, the C-PC/ $\beta$  had a minor inhibitory effect on growth of non-cancer cells (inhibitory ratios of C5/MJ and RPMI 1788 cells were 29.7% and 25.2% respectively, Figure 8). As a control, GST alone had no significant effects on the growth of any of the cell lines tested.

The morphology of cells can be important in many contexts. In culture, the morphology indicates the health status of the cells. We observed the morphologies of the different cells were changed greatly under the treatment of C-PC/ $\beta$ . We noticed that the morphology of cancer cells changed when the cells were treated with the C-PC/ $\beta$ . 686LN-M4C1 cells became non-transparent and distorted (Figure 9). Other cancer cell lines also changed significantly upon the treatment with C-PC/ $\beta$ . A majority of K562 cells changed from a round to a spindle-like morphology. HT29 cells grew sparsely. The 686LN cells became clustered and distorted. In contrast, no significant changes in morphology of non-cancer cells were observed under the same treatments.

### **Recombinant C-PC/ $\beta$ induced cancer cell apoptosis**

We speculated that the observed cell growth inhibition and cell morphology changes were due to induction of apoptosis by the C-PC/ $\beta$  treatment. To test the speculation, we examined apoptosis with all cancer and non-cancer cells using a commercially available apoptosis kit. Our experiments demonstrated that apoptosis was induced in three cancer cells upon the C-PC/ $\beta$  treatments. Highest level of apoptotic induction was observed with the metastatic head and neck cancer cells, 686LN-M4C1 (The enrichment factor of mono- and oligonucleosomes was approximately 4.0). As a control, GST alone did not induce apoptosis in any of the cells tested (Figure 10). No clear apoptosis was observed with non-cancer cells under the treatment of C-PC/ $\beta$ .

Activation of caspases are often associated with cell apoptosis [Landry, 2006]. The metastatic head and neck cancer cells 686LN-M4C1 suffered the highest degree of growth inhibition and apoptosis, we chose this cell line as our test system for probing caspase activity. Under the treatment of the C-PC/ $\beta$ , both Caspases -8 and -3 were activated as revealed by the cleavage of pre-Caspase-8 and pre-Caspase-3 (Figure 11). These results may suggest that the C-PC/ $\beta$  triggered 686LN-M4C1 cell apoptosis by activation of the extrinsic cell death pathway.

### **Cells were arrested in G0/G1 phase**

The cell cycle profiles were monitored 48 hours after C-PC/ $\beta$  treatment. An accumulation of cells with 2 N DNA content and a decrease in cells with 4 N DNA were evident by comparing to that of untreated cells (Figure 12). This result indicated that 686LN-M4C1 cells were mainly in the G0/G1 phase after the cells were treated with the C-PC/ $\beta$ . In addition, an increase in the number of cells with a sub-G1 DNA contents was clearly observed, which is a typical characteristic of cells under apoptosis (Figure 12). DNA fragmentation was produced during the

apoptosis process and some of them were eluted after the fixing and washing steps [Peng, 2005]. This observation provides an additional support for our conclusion that C-PC/ $\beta$  treatment induces apoptosis and cell cycle arrest.

### **Recombinant C-PC/ $\beta$ accumulated on plasma membrane**

The preceding experiments suggested that C-PC/ $\beta$  treatment inhibited cancer cell proliferation and induced apoptosis. C-PC/ $\beta$  treatment activated caspase and caused cell cycle arresting. To understand the molecular basis for the apoptotic induction and cell cycle arresting, we examined localization of the recombinant C-PC/ $\beta$  in cells after the treatment. We employed Immunofluorescent staining to detect the GST:C-PC/ $\beta$  in 686LN-M4C1 cells using antibody against GST. The recombinant GST:C-PC/ $\beta$  was mainly stained on the cancer cell plasma-membrane (Figure 13). While the membrane staining pattern was not observed with non-cancer cells. Interestingly, the level of accumulation of membrane C-PC/ $\beta$  correlated with the degree of cell proliferation inhibition (Compare Figure 13 to Figure 8). This observation suggested a potential role of plasma-membrane C-PC/ $\beta$  in cell proliferation inhibition.

### **Tubulin and GAPDH were the target proteins on the plasma membrane**

Since the recombinant C-PC/ $\beta$  proteins were mainly accumulated on the cancer cell membrane, we attempted to probe the protein or protein complex that interacted with C-PC/ $\beta$  in the plasma membrane. To this end, a GST-pull down experiment with membrane extracts made from 686LN-M4C1 cells using the GST:C-PC/ $\beta$  was carried out. A 55 kDa and a 36 kDa protein bands were among the other proteins that specifically associated with the GST:C-PC/ $\beta$  were found. (Figure 14). The protein bands were excised out. After the in-gel trypsin digestion, MALDI-(ms/ms) analyses (Figure 15) showed that the 55 kDa band was  $\beta$ -tubulin (gi|57209813, NCBI) and the 36 kDa band was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

(gi|31654, NCBI). The identity of the proteins was further verified by immunoblot (Figure 16). In addition, the interactions of C-PC/ $\beta$  with  $\beta$ -tubulin and GAPDH were further confirmed by co-immunoprecipitation experiments carried out using antibodies against  $\beta$ -tubulin and GAPDH with the cell extracts made from C-PC/ $\beta$  treated 686LN-M4C1 cells.

### **C-PC/ $\beta$ interacted with $\beta$ -tubulin on the cell membrane promoting depolymerization of microtubules**

Since the recombinant C-PC/ $\beta$  proteins were mainly accumulated on the cancer cell membrane, we attempted to probe the protein or protein complex that interacted with C-PC/ $\beta$  in the plasma membrane. We found that tubulin and GAPDH were specifically associated with GST:C-PC/ $\beta$ . First, we asked whether the cytoskeleton microtubules were affected by this interaction.

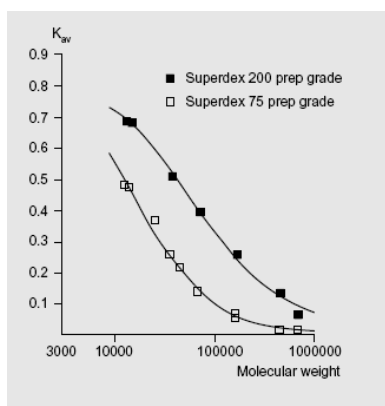
We examined ratio of depolymerized/polymerized tubulin by immunoblotting the soluble/insoluble tubulin in whole cell extract of the C-PC/ $\beta$  treated 686LN-M4C1 cells. The ratio of depolymerized/polymerized tubulin was around 0.949 for C-PC/ $\beta$  treated cells, and was 0.371 for PBS treated cells (Figure 17). This data suggests that there was a substantially higher level of soluble tubulin in C-PC/ $\beta$  treated cells compared to untreated cells. This was an indication that microtubules were depolymerized under the treatment of the  $\beta$  subunit of C-PC. Interestingly, we found that microfilaments were also depolymerized under the treatment of the C-PC/ $\beta$  by similar experimental approaches. The ratio of depolymerized/polymerized actin was around 0.721 for the C-PC/ $\beta$  protein treated cells and was 0.211 for PBS treated cells (Figure 17). Thus, our results demonstrated that C-PC/ $\beta$  interacts with  $\beta$ -tubulin on the cell membrane promoting depolymerization of cytoskeletons, and both microtubules and microfilaments.

### **C-PC/ $\beta$ interacted with GAPDH and promoted GAPDH nuclear translocation**

Interaction of C-PC/ $\beta$  with GAPDH is intriguing. Biological functions of GAPDH were dependent upon the subcellular localization. The nuclear form of GAPDH has been suggested to be a key transcriptional coactivator necessary for entry into S phase [Zheng, 2003; Sirover, 2005]. We have observed the effects of C-PC/ $\beta$  on cell cycle progression. The cells were arrested in the G0/G1 phase under the treatment of the C-PC/ $\beta$ . We suspected that C-PC/ $\beta$  treatment may have effects on GAPDH cellular localization. To test this idea, we examined the level of GAPDH in the cell nucleus and cytoplasm by immunoblotting the GAPDH in the nuclear extracts and whole cell lysate made from 686LN-M4C1 cells before and after the C-PC/ $\beta$  treatment. It was clear that the level of the nuclear GAPDH was greatly decreased after the incubation with the C-PC/ $\beta$  for 24 hours. Interestingly, GAPDH expression level was higher under the treatment of the C-PC/ $\beta$  as demonstrated via immunoblotting a whole cell lysate by antibody against GAPDH (Figure 18). This might be the result of feedback of the low level of the nuclear form of GAPDH.

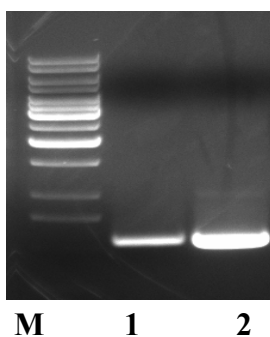
### **No difference in distribution of $\beta$ -tubulin and Histone H2A was observed under C-PC/ $\beta$ treatment**

Since the expression level of GAPDH was increased and the nuclear distribution was significantly reduced under the C-PC/ $\beta$  treatment. We wondered whether this phenomenon was specific to the GAPDH. To this end, we examined the nuclear level of  $\beta$ -tubulin and Histone H2a by the same experimental procedure. Our results showed that, both  $\beta$ -tubulin and Histone H2A did not change the expression level and cellular localization under the treatment of C-PC/ $\beta$  for 24 hours (Figure 19). We therefore conclude that C-PC/ $\beta$  treatment leads to nuclear export and up-regulation of GAPDH. The effects on the distribution and expression of GAPDH under the treatment of C-PC/ $\beta$  are likely specific.



**Graph 1** The selectivity curve for superdex 200 prep grade  
(Amershan Bioscience, data file of gel filtration media: Superdex 30, 72 & 200 prep grade BioProcess<sup>TM</sup> Media)

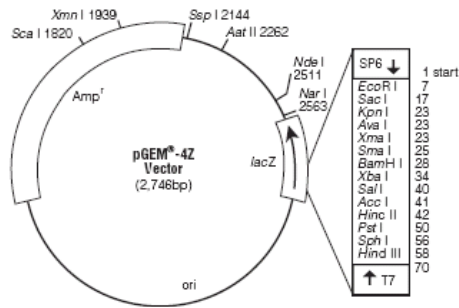
A selectivity curve is usually fairly straight over the range  $K_{av}=0.1$  to  $K_{av}=0.7$ , which is shown in **Graph 1**. The molecular weight range, which lies between these values, is defined as the useful fractionation range of the medium. Proteins with a molecular weight between 30,000–600,000Da MW are considered to be in the high selectivity range for Superdex 200 prep grade. The molecular weights of GST- $\beta$ , GST, and  $\beta$  are in this range.



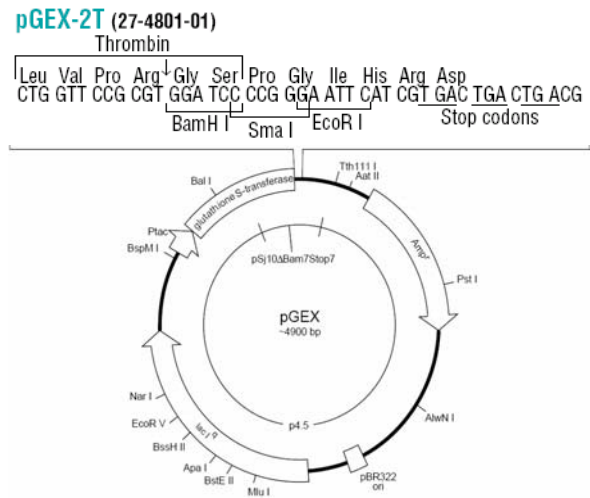
**Figure 1** Whole cell PCR product for  $\beta$  subunit of C-PC.

**Lane 1 and lane 2** were the PCR products of  $\beta$  for vector pGEX-2T; *Anabaena* PCC 7120 alga was used. 0.5 Kb DNA fragments were obtained for each product. The amplified DNA product contains BamHI and EcoRI sites, which were used to clone into the pGEX-2T vector.

a.



b.



**Figure 2** Vectors.

(a) Map of pGEM-4Z; (b) Map of pGEX-2T. This figure shows the maps of two vectors used. pGEX-2T was used to express the subunit of C-PC. In the pGEM-4Z vector, the Sma I recognition site in the multiple cloning region was used to clone the PCR product; in pGEX-2T vector, the BamH I and EcoR I recognition sites were used to sub-clone the PCR products. pGEX-2T is an expression vector for fusion protein, which encodes glutathione S-transferase gene at the N-terminus of the fused protein; there is also a thrombin recognition site between the GST and the fused protein.



**Table 1** The amino acid sequences of  $\beta$  subunit of C-PC (*Anabaena* 7120) and GST (pGEX-2T).

|                         |   |
|-------------------------|---|
| $\beta$ subunit of C-PC | GSmtldvftkvvsqadsrgefslsneqlaldanvvkegnkrldvvnritsnasaiv<br>tnaaralfeeqpqliapgggnaytnrrmaaclrdmeilryvtyailagdasvlddrc<br>lnglretyqalgtptgssvavgvqkmkdaavgiandpngitkgdcsqlisevasyf<br>draaaavg   |
| GST                     | mspilgywkikglvqprrllleyleekyeehlyerdegdwknkkfelglefpnlp<br>yyidgdvkltsmaiiryiadkhnmlggcpkeraeismlegavldirvgvsriay<br>skdfetlkvd klslpmlkmfedrlchkylnghvthpdmlydaldvvly<br>mdpmcldafpklvckkriaipqidkylksskyiawplqgwqatfggdhpk<br>sdlvprgsrrasvgsphrd |

[www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=38893](http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=38893) (subunits of C-PC)

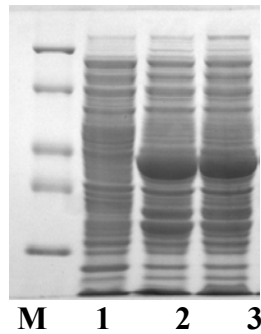
[www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=345178](http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=345178) (GST)

The published gene sequences of the subunits of C-PC were obtained from Pubmed (No. X05239). The DNA sequence of GST was also obtained from Pubmed (No. A01438). Note: The capital amino acids in the sequence of  $\beta$ /C-PC were from pGEX-2T vector.

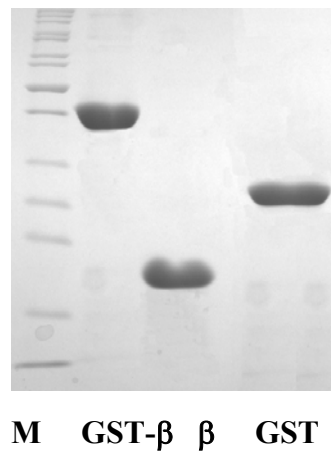
**Table 2** Theoretical analysis of the proteins.

| Protein      | Molecular weight | PI   | Hydropathicity | Transmembrane helices prediction (topology analysis) | Stability |
|--------------|------------------|------|----------------|--|-----------|
| GST          | 28165.7          | 7.05 | -0.385         | no   | stable    |
| GST- $\beta$ | 46678.6          | 6.22 | -0.234         | no   | stable    |
| $\beta$      | 18530.9          | 5.0  | -0.025         | no   | stable    |

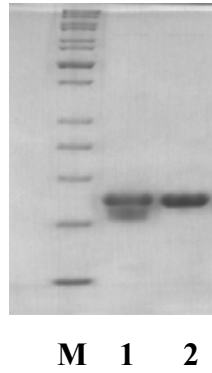
Modern transmembrane prediction and Protparam (Swiss-Prot/TrEMBL) soft analysis on the website (<http://www.expasy.org/>) were used to analyze the theoretical molecular weight, PI, hydropathicity, transmembrane helices predicted, and stability level of each protein.



**Figure 3** Expression of the recombinant GST tagged  $\beta$  subunit of C-PC in *E. coli* BL 21. Bacterial cell lysates were analyzed in 10% SDS-PAGE, before IPTG induction (**lane 1**: GST:C-PC/ $\beta$ ); after IPTG induction (**lanes 2, 3**: GST:C-PC/ $\beta$ ).



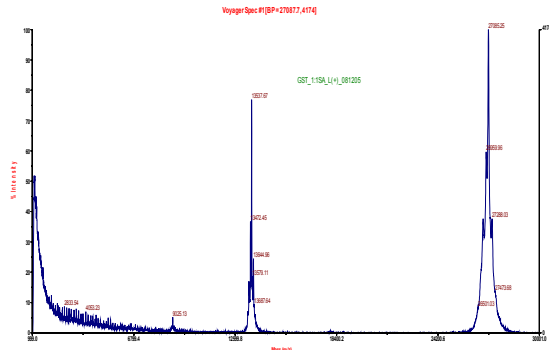
**Figure 4** Purified proteins of GST:C-PC/ $\beta$ ,  $\beta$  and GST. The recombinant proteins (GST- $\beta$ ,  $\beta$  and GST) were further purified by gel filtration: Sephadex-200. Purified recombinant proteins were analyzed by 10% SDS-PAGE gel. **Lane 1**: recombinant GST:C-PC/ $\beta$ ; **lane 2**: recombinant  $\beta$ ; **lane 3**: recombinant GST



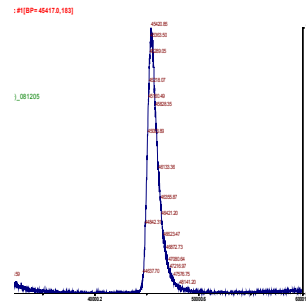
**Figure 5** Dialysis  $\beta$  subunit of C-PC.

The  $\beta$  subunit of C-PC was dialyzed; and a 10% SDS-PAGE gel was used to analyze the recombinant proteins. **Lane 1**, the  $\beta$  subunit of C-PC was dialyzed in the dialysis buffer without reducing agent added; **lane 2**, the  $\beta$  subunit of C-PC was dialyzed in the dialysis buffer with reducing agent added.

a,

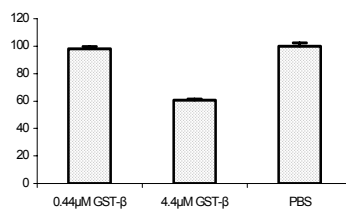


b,



**Figure 6** Mass spectrometry of GST and GST:C-PC/ $\beta$ .

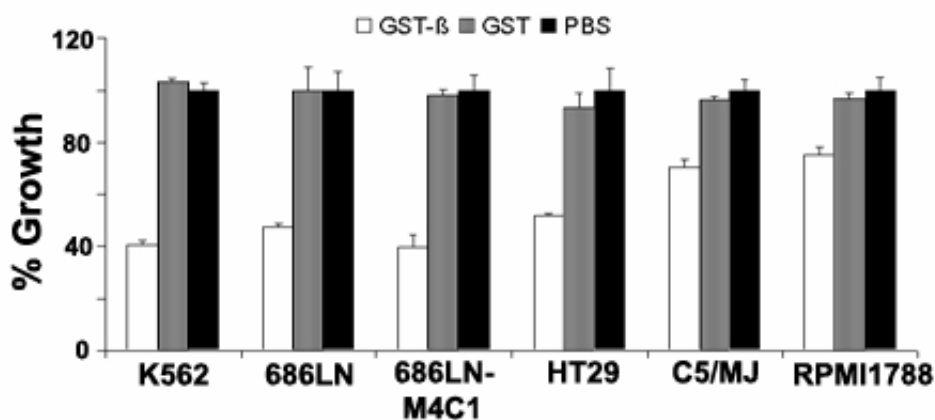
Molecular weights of the obtained recombinant proteins were determined by MALDI-TOF mass spectrometry. **(a)**, mass spectrometry of GST, which indicated that the molecular weight of the recombinant GST is 27.1 kDa; **(b)**, mass spectrometry of GST:C-PC/ $\beta$ , which showed that the molecular weight of the recombinant GST:C-PC/ $\beta$  is 45.4 kDa.



**Figure 7** Proliferation of K562 cells treated with different doses of GST:C-PC/β.

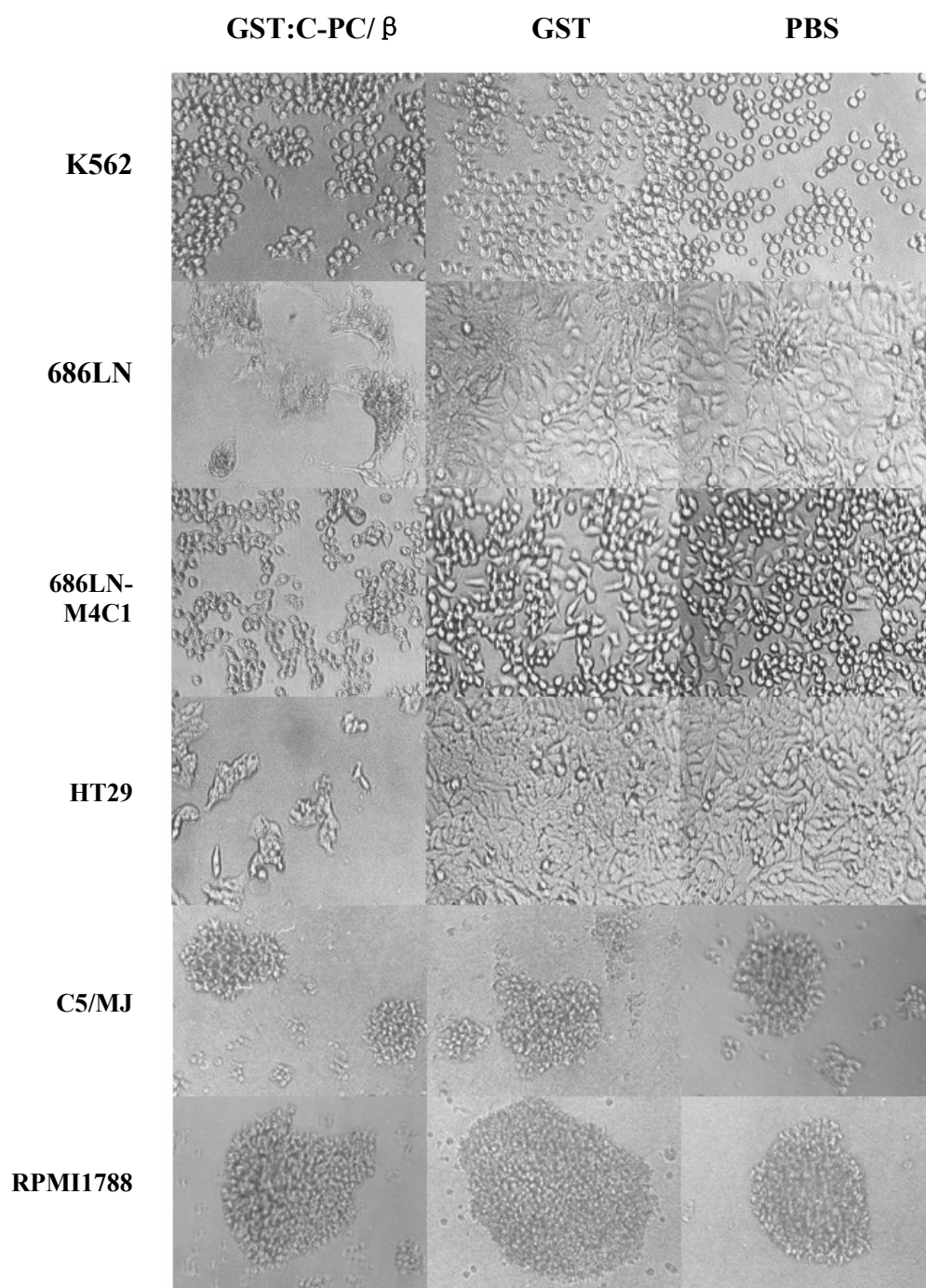
MTT assays of cell proliferation of K562 cells treated with different doses of GST:C-PC/β.

The optimum dosage of the protein to the K562 cell was around 5 μM. The cell proliferations were expressed as percentage of growth and were calculated in comparison to PBS treated cells. The number of cells in the control was taken as 100%. Values were calculated as mean  $\pm$  S.D. of triplicate measurements ( $P < 0.05$ ).



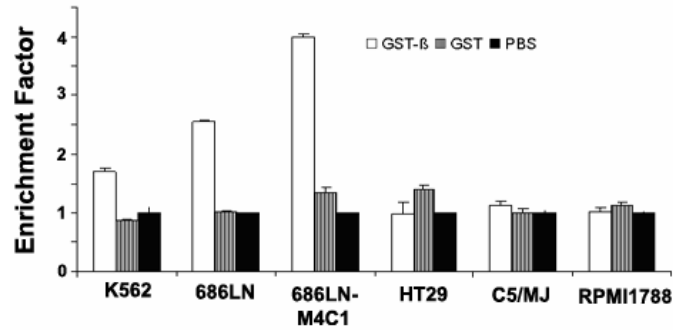
**Figure 8** Proliferation of six cell lines under the treatment of 5 μM of GST:C-PC/β.

MTT assays of cell proliferation of different cell lines (indicated) treated with different agents (indicated). The treatment of GST was used as a control. The results suggested that 5 μM of the recombinant C-PC/β inhibits the growth of cancer cells greatly.



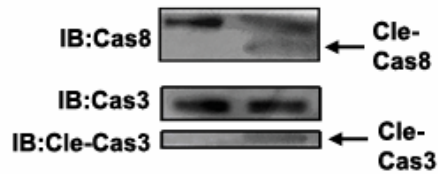
**Figure 9** Morphology of the cells under different treatments.

Morphologies of different cell lines (indicated) upon the treatments of 5 $\mu$ M of GST:C-PC/ $\beta$  or other agents (indicated). GST (5 $\mu$ M) and PBS treatments of different cells were used as a control.



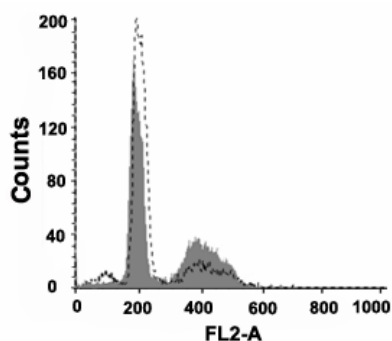
**Figure 10** Apoptosis assay of six cell lines under different treatments.

Apoptosis of different cell lines (indicated) upon treatment with different agents (5 $\mu$ M of GST:C-PC/ $\beta$ , 5 $\mu$ M of GST, and PBS ). Apoptosis was expressed as the enrichment of mono- and oligonucleosomes released into the cytoplasm.



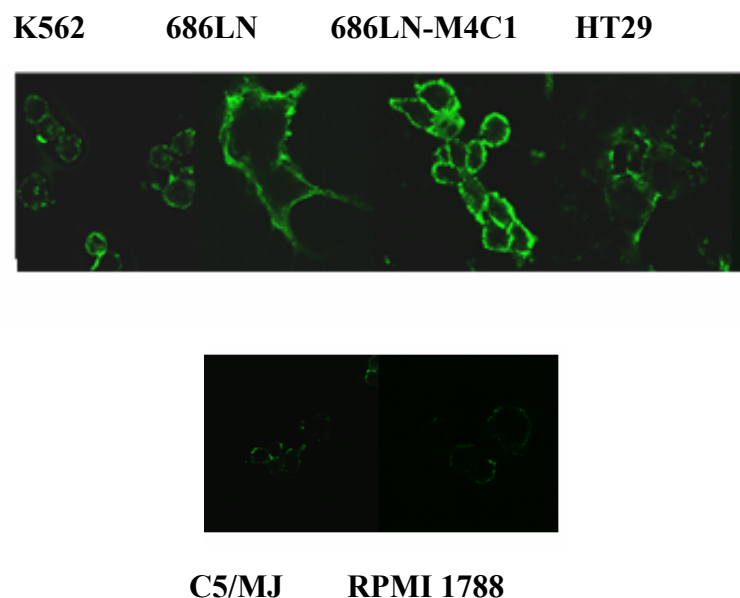
**Figure 11** Immunoblot analyses of the activities of Caspase-3 and Caspase-8

Immunoblot analyses of cellular Caspase -3 and -8 in cell extracts made from 686LN-M4C1 cells that were treated with recombinant C-PC/ $\beta$  or PBS using antibodies against Cas-3 or Cas-8. The arrows indicate the cleavage products (Cle-Cas 8, and Cle-Cas 3).



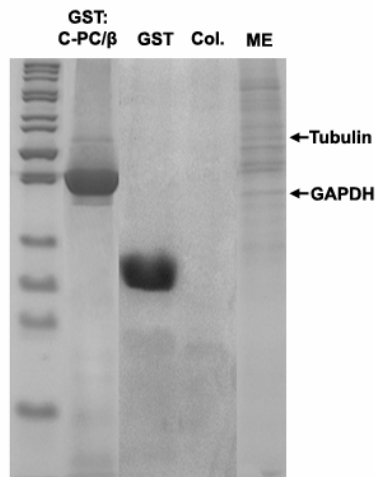
**Figure 12** Cell cycle analysis of 686LN-M4C1 after the treatment of 5 $\mu$ M of recombinant C-PC/ $\beta$

FACS analyses of cell cycle of 686LN-M4C1 cells upon the treatment of PBS (solid line) or C-PC/ $\beta$  (dot line). An accumulation of cells with 2N DNA contents and a decrease in cells with 4N DNA was evident by comparing to that of untreated cells. An increase in cells with a sub-G1 DNA content was clearly observed.



**Figure 13** Immunofluorescent staining of GST:C-PC/ $\beta$  in different cell lines upon 5 $\mu$ M GST:C-PC/ $\beta$  treatment.

Immunofluorescent staining of GST:C-PC/ $\beta$  in different cell lines (indicated) upon 5  $\mu$ M GST:C-PC/ $\beta$  treatment using antibody against GST under confocal technique. The green signal represents staining of GST:C-PC/ $\beta$ .



**Figure 14** GST pull down experiment and SDS-PAGE analysis.

SDS-PAGE analysis of GST:C-PC/  $\beta$  pull-down from membrane extracts made from 686LN-M4C1 cells. The proteins were pulled down by GST:C-PC/  $\beta$  (IP). The GST moiety, the column (Col.), and the membrane extracts (ME) were used as controls. Arrows indicate the protein bands that associate with C-PC/  $\beta$ .

tubulin, beta pol...[gi:57209813] chromosome="6"

mfwevisdehgidptgtyhgdsdlqldrisvyneatggkyvprailvdlepgtmdsvrsgpfqgqifrpdnfvfgqsgagnnwakghytegae  
 lvdsvldvvrkeaescdclqgqqlthslgggtgsgmgltliskireevpdrimntfsvvpspkvsdtvvepynatlsvhqlventdetycidnealydi  
 cfrtlkltpygdlnhlvsatmsgvttclrfpgqlnadlrklavnmvvpfprlhffmpgfapltsgsqgyraltvpeltqqvfdaknmmaacdpr  
 hgryltvaavfrgrmsmkevdeqmlnvqknssyfvevipnnvktavcdipprglkmavtfignstaiqlfkriseqftamfrkaflhwyte  
 gmdemefteaesnmndlvseyqqyqdataeeedfgeaeaea  
 33.8% match: 144/426

**GAPDH sequence**

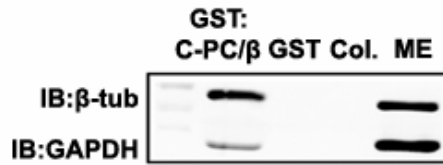
mgkvkvvgngfgrigrivtraafnsgkvdivaindpfidlnymvymfydsthgkfhgtvkaengklvingnpitifqerdpskikwgdagaey  
 vvestgvftmekagahlqggakrviisapsadapmfvmgvnhekydnlkiisnascttnclaplakvihdnfgiveglmttvhaitatqktvdgps  
 gklwrddrgalqniipastgaakavgvvipeldgkltgmafrvptanvsvvdltrlekpakyddikkvvkqasegplkgilgytehqvssdfns  
 dthsstfdagagialndhfvliswydnefgysnrsvdlmahmaske  
 25.1% match: 84/335

**Figure 15** MALDI/TOF/TOF-MS analyses of the C-PC/  $\beta$  association proteins.

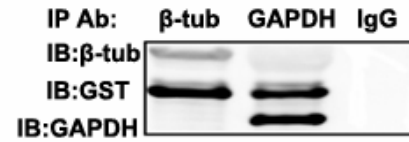
MALDI/TOF/TOF-MS analyses of the proteins, which were pulled down by GST:C-PC/  $\beta$ . The whole amino acid sequences were obtained from NCBI. Bold characters indicate the sequences identified by the mass-spectroscopy.



a, \_



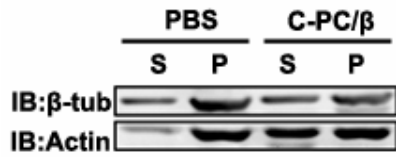
b,



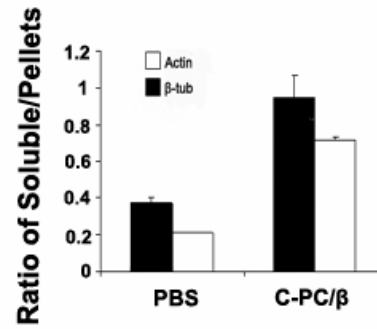
**Figure 16** Immunoblot and co-immunoblot methods to further prove the interaction between C-PC/β and β-tubulin or GAPDH.

**(a)** Immunoblot analyses of the GST:C-PC/β pull-downs by immunoblot using antibodies against β-tubulin (IB:β-tubulin) and against GAPDH (IB:GAPDH). **(b)** Co-immunoprecipitation of GST:C-PC/β by the antibodies (IP: Ab) against β-tubulin (β-tub) and GAPDH (GAPDH) was analyzed by immunoblot the co-precipitates using appropriate antibodies (indicated). IP by mouse IgG was a control for the co-immunoprecipitation.

a,



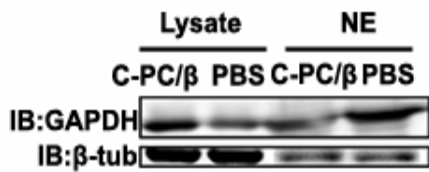
b,



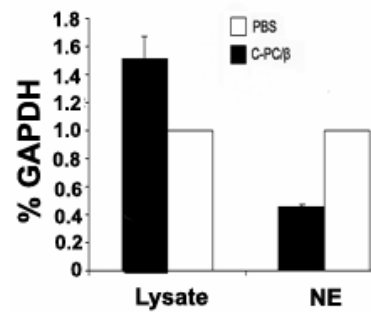
**Figure 17** Soluble and assembled tubulin/ actin measurement via immunoblot method.

Immunoblot analyses of soluble (S) tubulin and actin and assembled (P, precipitates) tubulin and actin in the soluble and insoluble extracts made from the PBS or C-PC/β treated 686LN-M4C1 cells (**upper panel**). The lower panel shows the quantification of the immunoblotting results expressed as ratio of soluble/insoluble tubulin (filled bars) and actin (open bars).

a,



b,



**Figure 18** Expression level and subcellular distribution of GAPDH via immunoblot method.

a) Immunoblot analyses of cellular (**lysate**) and nuclear (**NE**) amounts of GAPDH in PBS or C-PC/ $\beta$  treated 686LN-M4C1 cells. IB of  $\beta$ -tubulin is used as a loading control. b) The lower panel shows the quantification of the immunoblotting results expressed in comparison to PBS treated results. The quantifications were expressed as relative GAPDH levels in nuclear extracts or in total cell lysate by defining the GAPDH levels in nuclear extracts or in total cell lysate made from PBS treated cells as 1(open bars).



**Figure 19** Distribution of  $\beta$ -tubulin and Histone H2A under C-PC/ $\beta$  treatment.

Immunoblot analyses of cytoplasmic (CE) and nuclear (NE) levels of  $\beta$ -tubulin and Histone H2A in PBS or C-PC/ $\beta$  treated 686LN-M4C1 cells. No changes in distribution of  $\beta$ -tubulin and Histone H2A were observed.

## DISCUSSION

A high yield of the recombinant proteins in *E. coli* BL-21, means subunit of C-PC are suitably expressed in *E. coli* bacteria (in the text, only beta subunit of C-PC was shown). This will help us study the characteristics of the subunits of C-PC. Both blue-green algae and *E. coli* belong to Eubacteria (same codon bias). They may share the similar protein modification systems. The recombinant protein should be modified the same way as the native protein. This study suggests that *E. coli* can be used to express and analysis other proteins from blue-green algae.

The molecular weight difference between theoretical molecular weight and MS analysis results may be caused by the purified protein not forming the disulphate bridge. The refolded protein sample treated with loading buffer without reducing reagent shows two bands (Figure 5, lane 1). It suggests that the bold band is the linear protein, which might be oxidized by the SDS contained in the gel, while the faint band still contains the disulphate bridge. The refolded protein sample treated with loading buffer (contained reduced reagent) (Figure 5, lane 2) ran as fast as the bold band (Figure 5, lane1). It is quite possible that the disulphate bridge of the protein, under the treatment of both reducing reagent and SDS, was totally oxidized. Therefore, only one band was observed (Figure 5, lane 2). It is not known whether the formation of the disulphate bridge will affect the pharmacological characteristics of the  $\beta$  proteins.

Based on the proliferation assays, C-PC/ $\beta$  has a higher inhibitory effect on highly metastatic cancer cells (686LN-M4C1 and K562) than on other cancer cells can be concluded.

GST had no significant proliferation inhibitory effect on all the cell lines under the treatment, which means the functional domain of the fusion protein is C-PC/ $\beta$ . Importantly, C-PC/ $\beta$  showed much lower proliferation inhibition effect on non-cancer cells than on cancer cells.

Though all the cancer cell lines showed cell proliferation inhibition under the treatment of recombinant C-PC/ $\beta$ , the apoptosis level of different cells does not correlate to the proliferation inhibition level. HT29 cells showed almost no apoptosis. As shown in the morphological characteristic (discussed in the last chapter), no obvious HT29 cell necrosis had occurred under the treatment. C-PC/ $\beta$  might induce growth stress on HT29, which slowed down the growth rate of the cells. No apoptotic phenomenon was observed among non-cancer cells. C-PC/ $\beta$  might slow down the growth rate of the non-cancer cells as well.

Both Caspase-8 and Caspase-3 were observed to be activated under the treatment of C-PC/ $\beta$ . While the pull-down results showed that cell death receptor may not directly targeted by the recombinant proteins. However, the target proteins (as indicated in the latter experiments) may recruit adaptor protein(s) on the cytoplasmic side. The adaptor protein, in turn, recruits Caspase-8 to trigger the death-inducing signal pathway. Caspase-8 will then be activated and is now able to directly activate Caspase-3, an effector protein, to initiate degradation of the cell.

It is intriguing that C-PC/ $\beta$  treatment activates Caspase-3 and -8, which subsequently triggers cell apoptosis. It was reported that the biliprotein C-PC purified from blue-green algae also induces cell apoptosis [Pardhasaradhi, 2003; Reddy, 2003; Subhashini, 2004]. However, the mechanism by which the C-PC triggers apoptosis seems different from the mechanism by which C-PC/ $\beta$  induces apoptosis. The C-PC enters the cytoplasm of cancer

cells [Subhashini, 2004], while the recombinant C-PC/ $\beta$  was mainly accumulated on the plasma membrane of K562 cells. On the other hand, under the same treatment of C-PC or C-PC/ $\beta$ , different cell lines show different responses. This phenomenon was reflected in previous studies, in which C-PC was used as the treatment agent. Activation of Caspase-8 was detected in Hela cells [Li, 2005], while not in K562 cells [Subhashini, 2004]. Activation of Caspase-8 was observed under C-PC/ $\beta$  treatment in our experiment. Different cell lines showed different treatment responses were also observed in this study. Under the C-PC/ $\beta$  treatment, cytoplasm GAPDH might be associated with actin and trigger the depolymerization of the actin. There is no evidence to demonstrate that C-PC has any effect on cytoplasm or nuclear GAPDH. This study suggests that the recombinant  $\beta$  subunit of C-PC works more effectively in inhibition of cell proliferation and apoptosis induction than the purified C-PC does.

686LN-M4C1 cells were mainly in the G0/G1 phase after the cells were treated with the C-PC/ $\beta$ . C-PC/ $\beta$  may interact with specific protein(s) that affect DNA replication and block the cell from moving into S-phase. The important question is where does C-PC/ $\beta$  interact with the cells. Another question is that, which protein(s) was (were) the interactive target for C-PC/ $\beta$ .

C-PC/ $\beta$  was mainly accumulated on the cell plasma membrane, which is consistent with the predicted property of C-PC/ $\beta$  proteins (no transmembrane domain was predicted). On the other hand, C-PC/ $\beta$  has low accumulation level on non-cancer cell surfaces. This may due to the membrane proteins have different structures/protein complexes between cancer cells and non-cancer cells.

One question is that whether the extract detergent disrupted the interaction between GAPDH/tubulin and the cell death receptor. On the other hand, the trace amount of proteins in the loading sample was not visible in SDS-PAGE gel. Therefore, GAPDH and tubulin might not be the only proteins associated with C-PC/ $\beta$ . Promisingly, both GAPDH and tubulin play an important role in the toxic effects on the cancer cells.

The main disadvantage of traditional IP and Co-IP is that the conditions used to elute the precipitated antigen also released the antibody, contaminating the antigen and destroying the antibody support. This phenomenon was reflected in our Co-IP results:  $\beta$ -tubulin has a similar molecular weight as the heavy chain, which caused the immunoblot result of  $\beta$ -tubulin to be quite faint (Figure 16 b).

Tubulin and GAPDH were the associated proteins for C-PC/ $\beta$  on the plasma membrane. Therefore, we focused on these two proteins and tried to find the mechanism how they play a role in apoptosis.

GAPDH functions as a glycolytic enzyme within the cytoplasm, but beside its metabolic function it is involved in early steps of apoptosis, which trigger the translocation of GAPDH into the nucleus [Dastoor, 2001]. Interestingly, our results showed that, under treatment with C-PC/ $\beta$ , a greater amount of GAPDH was in the cytoplasm, and subsequently led to the apoptosis. A previous report showed that GAPDH expression is three times greater in apoptotic cells compared to non-apoptotic ones. The results also showed that GAPDH was over expressed during the treatment with C-PC/ $\beta$ . Both over-expression and abnormal distribution of the GAPDH inside of the cell might be the main reasons for the programmed cell death. C-PC/ $\beta$  cause 686LN-M4C1 cells to arrest at G0/G1 phase of cell cycle. The data

showed that C-PC/ $\beta$  treatment led to a great amount of GAPDH localization inside the cytoplasm. It is conceivable that low levels of nuclear GAPDH induced by C-PC/ $\beta$  treatment cause the cell cycle arrest, which subsequently inhibited cell proliferation. How the C-PC/ $\beta$  reduces nuclear GAPDH level is an open question. The speculation is, during treatment with the C-PC/ $\beta$  proteins, GAPDH is targeted by C-PC/ $\beta$  and recruited to the plasma membrane. This recruitment promotes nuclear export of GAPDH. It has been shown that if the amount of GAPDH in the nucleus is lowered to a certain level, it then inhibits the cells' ability to enter into S phase [Zheng, 2003]. Lower levels of nuclear GAPDH will in turn activate the expression of GAPDH.

The roles of actin and tubulin in tumor cell blebbing (apoptosis) have been reported [Ostrowski, 2005; Vilpo, 2000]. This study showed that both actin filaments and microtubules were depolymerized under the treatment of C-PC/ $\beta$ . Also, 686LN-M4C1 cells were induced into apoptosis. It is believable that the C-PC/ $\beta$  inhibits cell proliferation and promotes apoptosis by triggering cytoskeleton depolymerization and activation of Caspases that are associated with the extrinsic apoptosis pathway. Actin filaments were also reported to be associated with GAPDH under stressful environments [Schmitz, 2002]. The depolymerization of actin might result from the association with GAPDH under the apoptotic induction by C-PC/ $\beta$ . Depolymerization of actin might also be the result of the apoptosis.

The tubulin was depolymerized during the treatment of C-PC/ $\beta$ , while there was no significant change of expression and cellular distribution of tubulin. It might be concluded that the proliferation and apoptosis of 686LN-M4C1 only related to the polymerization status of the tubulin. Histone H2A showed no distribution inside the cytoplasm: this showed that



cytoplasmic protein solution had not been contaminated by the nuclear protein. With twenty-four hours of treatment with C-PC/ $\beta$ , almost no degraded DNA was released into the cytoplasm and no Histone H2A was observed inside the cytoplasm.

Tubulin is one of the intracellular molecular targets of many anticancer drugs, such as Colchicine (C9754, Sigma), Nocodazole (M1404, Sigma), and Paclitaxel (T7191, Sigma). Tubulin-binding agents that cause rapid depolymerization of cytoskeletal microtubules show promise as cancer targeting agents. GAPDH has been implicated in certain neurological diseases: GAPDH is able to bind to the gene products from neurodegenerative disorders such as Huntington's disease, Alzheimer's disease, Parkinson's disease, and Machado-Joseph disease through stretches encoded by their CAG repeats. The interaction of GAPDH with disease-related proteins suggests that it is a potential molecular target for drug development [Chuang, 2005]. Both tubulin and GAPDH associated on the cell plasma membrane were the targets for the C-PC/ $\beta$ . C-PC/ $\beta$  is a good binding agent for both tubulin and GAPDH. Previous results showed that C-PC entered K562 cells under incubation with C-PC; however, the  $\beta$  subunit of C-PC was not easily released from the whole C-PC [Duerring, 1991]. Whether this anti-cancer property is more effective if C-PC/ $\beta$  is delivered inside of the cancer cell is not known. Therefore, it will be a good idea to introduce the C-PC/ $\beta$  into the cytoplasm to test the anti-cancer effects.

## APPENDICES

### Chemicals

acrylamide/bisacrylamide solutions: Fisher BioReagent  
acetonitrile (CAN): VWR International  
acetic acid: VWR International  
AEBSF(4-(2-Aminoethyl)benzenesulphonyl fluoride): Calbiochem  
agarose: MP Biomedicals  
ammonium persulfate (APS): Sigma Aldrich  
ampicillin solutions: Teknova  
Bio-Rad protein assay: Biorad Laboratories  
boric acid: Biorad Laboratories  
bovine serum albumin (BSA): Promega  
bromophenol blue: EMD Biosciences  
 $\beta$ -mercaptoethanol: Sigma Aldrich  
 $\text{CaCl}_2$ : Sigma Aldrich  
citric acid: Fluka  
Coomassie Blue: Sigma Aldrich  
cyanogen bromide: Sigma Aldrich  
DAPI (4',6'-diamidino-2-phenylindole): Sigma Aldrich  
DMSO (dimethyl sulphoxide): Pierce  
DTT (dithiothreitol): Shelton Scientific  
DMEM (Dulbecco's Modified Eagle's Medium): HyClone  
dNTP mixture: Takara Mirus Bio  
EGTA (ethylene glycol bis-N,N,N',N'-tetraacetic acid): Sigma Aldrich  
ethanol: AAPER Alcohol & Chemical  
EB (ethidium bromide): Sigma Aldrich  
EDTA (ethylenediaminetetra-acetic acid): Sigma Aldrich  
formic acid: EMD Biosciences  
FCS (fetal calf serum): HyClone  
formaldehyde: Calbiochem  
gelcode® blue stain reagent: Pierce  
glutathione: Sigma Aldrich  
glutathione-agarose: Sigma Aldrich  
glycerol: Sigma Aldrich  
glycine: MP Biomedicals  
isopropanol: VWR International  
IPTG (isopropyl-beta-D-thiogalactopyranoside): Sigma Aldrich  
image-iT™ FX signal enhancer: Molecular Probes  
lysozyme: Sigma Aldrich  
KCl: Fisher Bioreagent  
 $\text{KH}_2\text{PO}_4$ : Fisher Bioreagent  
 $\text{MgCl}_2$ : Fisher Bioreagent

methanol: VWR International  
NaCl: Fisher Bioreagent  
NaHCO<sub>3</sub>: Fisher Bioreagent  
Na<sub>2</sub>HPO<sub>3</sub>: Fisher Bioreagent  
NaH<sub>2</sub>PO<sub>3</sub>: Fisher Bioreagent  
TEMED (N,N,N',N'-tetramethylethylene diamine): Sigma Aldrich  
NaOH: Fisher Bioreagent  
NP-40: Roche Applied Science  
N-Lauroyl sarcosine (C<sub>15</sub>H<sub>28</sub>NO<sub>3</sub>Na): Sigma Aldrich  
oxidized glutathione: Sigma Aldrich  
paclitaxel: Sigma Aldrich  
paraformaldehyde: Sigma Aldrich  
penicillin: Mediatech  
PFU and PFU buffer: Sigma Aldrich  
phenol/chloroform: Promega  
PMSF (phenylmethylsulfonyl fluoride): Fluka  
PI (propidium iodide) /RNase staining buffer: BD Bioscience  
protease inhibitor cocktail: Sigma Aldrich  
RPMI 1640 medium: HyClone  
reduced glutathione: Sigma Aldrich  
RIPA buffer: Sigma Aldrich  
SDS (sodium dodecylsulfate): Fisher BioReagent  
streptomycin: Mediatech  
thrombin: Sigma Aldrich  
trypsin-EDTA: Cellgro  
tryptone: Sigma Aldrich  
syringe filter (0.45 µm): Sigma Aldrich  
Tris (tris-(hydroxymethyl)-aminomethane): Sigma Aldrich  
Triton X-100: Sigma Aldrich  
Tween-20: Sigma Aldrich  
urea: Fisher Biotech  
yeast extract: Sigma Aldrich

### **Enzymes and Antibodies**

Sma I: Fermentas  
BamH I: Fermentas  
EcoR I: Fermentas  
T4 Polynucleotide Kinase: Promega  
T4 DNA ligase: Fermentas  
Actin monoclonal Ab: SC-8432 Santa cruz biotechnology  
β-tubulin monoclonal Ab: Sigma Aldrich  
β-tubulin polyclonal Ab: abcam  
GAPDH monoclonal Ab: Chemicon  
GAPDH polyclonal Ab: abcam

GST monoclonal Ab: Amersham Biosciences  
Histone polyclonal Ab against Histone H2A: Cell Signaling  
mouse monoclonal Ab of anti-caspase-3: Cell Signaling  
mouse monoclonal Ab of anti-caspase-8: Cell Signaling  
rabbit monoclonal Ab of anti-cleaved-caspase-3: Cell Signaling

#### **Plasmids and cell lines**

pGEM-4Z: Promega  
pGEX-2T: Pharmacia  
Anabaena PCC 7120: UTEX  
*E. coli* BL-21: Stratagene  
JM 109: Promega  
C5/MJ: T lymphocyte (ATCC)  
HT 29: colorectal adenocarcinoma (ATCC)  
K562: Human chronic myeloid leukemia cells (ATCC)  
686LN: 686LN was derived from a squamous cell carcinoma of the head and neck patient's metastatic tumor (a gift from George Z. Chen in Amory University)  
686LN-M4C1: 686LN-M4C1 was derived from 686LN cells passaged four times in nude mice (a gift from George Z. Chen in Amory University)  
RPMI 1788 cells: B lymphocyte (ATCC)

#### **Laboratory equipments**

AKTA Basic 10 FPLC System: Amersham Bioscience  
Allegra<sup>TM</sup> 6R Centrifuge: Beckman Coulter  
C25 Indubator Shaker: NewBrunswick Scientific  
FACS (Becton Dickinson): GE Healthcare  
EasyCast<sup>TM</sup> Horizontal Electrophoresis System: Owl Separation System  
EasyCast<sup>TM</sup> Vertical Electrophoresis System: Owl Separation System  
EpiChemi<sup>3TM</sup> Darkroom Bioimaging System: UVP, Inc.  
Eppendorf Centrifuge 5415D: Eppendorf AG  
Eppendorf Mastercycler: Eppendorf AG  
Gel Dryer: Fisherscientific  
Mini-PROTEAN@II Eletrophoresis Cell: Biorad Laboratories  
Mini Trans-Blot Cell: Biorad Laboratories  
NanoPure Diamond<sup>TM</sup> Water System: Barnstead International  
NuAire<sup>TM</sup> CO<sub>2</sub> Water-Jacketed Incubator: NuAire  
Phosphoimager BAS 1500: FUJIFILM Medical Systems  
Precision DuraFuge 100: Precision  
RC-5C PLUS Superspeed Refrigerated Centrifuge: Thermo Electron Corporation  
Rotomix® Variable Speed Rotator: Barnstead International  
Sonic Dismembrator 500: Fisherscientific  
UV-1700 UV-Visible Spectrophotometer: Shimadzu Corporation  
Vacufuge® Concentrator 5301: Eppendorf AG  
Victor<sup>3</sup>V 1420 Multilabel Counter: Perkin Elmer

VistaVision Microscope: VWR International

**Reagents, Kits and other materials:**

Alexa Fluor 488 goat anti-mouse IgG (H+L): Molecular Probes  
Immunopure Protein A agarose: Molecular Probes  
Prolong Gold anti-fade reagent: Molecular Probes  
Cell Death Detection ELISA<sup>PLUS</sup> Kit: Roche  
CellTiter 96® non-Radioactive Cell Proliferation Assay: Promega  
Image-iT<sup>TM</sup> FX Kit: Molecular Probes  
Mem-PER® Eukaryotic Membrane Protein Extraction Reagent Kit: Pierce  
Nuclear extraction Kit: Active Motif  
ProteoProfile<sup>TM</sup> Trypsin In-Gel Digest Kit: Sigma Aldrich  
ProteoSilver<sup>TM</sup> Plus Silver Stainin Kit: Sigma Aldrich  
QIAquick PCR Purification Kits: QIAGEN  
QIAprep® Spin Miniprep Kits: QIAGEN  
QIAquick Gel Extraction Kit: QIAGEN  
Rapid DNA Ligation Kit: Fermentas  
Centriprep Centrifugal Filter Devices: Amicon  
culturewell<sup>TM</sup> chambered cover-glass: Molecular Probes  
multichannel pipette: Eppendorf  
nitrocellulose: Optitran BA-S83, Schleider & Schuell  
Sephedex 200 gel-filtration column: Amershan Bioscience  
Siliconized Eppendorf tube: Sigma Aldrich  
sterile 96-well cell culture plates: Promega  
syringe filter (0.22 µm): Sigma Aldrich  
Paraffin-wax-coated matrix-assisted laser desorption/ionization (MALDI) target plate: Ion  
Channel Media Group  
ZipTip C18 tips: Millipore

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## LIST OF PUBLICATIONS

1. Wang, H.; Liu, Y.; Gao, X.; Carter, C. L.; Liu, Z. R., The recombinant beta subunit of C-phycocyanin inhibits cell proliferation and induces apoptosis. *Cancer Lett* 2007.
2. Yang, L.; Lin, C.; Zhao, S.; Wang, H.; Liu, Z.R., Phosphorylation of p68 RNA helicase plays a role in platelet-derived growth factor-induced cell proliferation by up-regulating cyclin D1 and c-Myc expression. *J Biol Chem.* 2007.

